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PRINCIPAL INVESTIGATOR: F. Carter Bancroft, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai Medical Center
New York, New York 10029-6574

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F. Carter Bancroft, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai Medical Center
New York, New York 10029-6574

E-Mail: carter.bancroft@mssm.edu

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Purpose of project is to clone, isolate and characterize protein markers specifically expressed by advanced stage, androgen-independent prostate cancers. Two types of markers with this specificity are targeted: proteins secreted specifically by advanced stage prostate cancer cells, potentially useful in serum-based assays for these tumors; markers expressed specifically on the surface of these cells, potentially useful as advanced prostate cancer. Work during the period covered included: (i) Incorporation of the powerful new DNA Microarray Technology into the project, and its use to investigate differential gene expression in the LNCaP series of human cell lines, representing different stages of prostate cancer. (ii) Studies demonstrating potential utility but insufficient purity of membrane fraction (MF) prepared from these cells; and use of an alternative fractionation scheme, successful only with the most advanced cells (C4-2B). We plan to complete the studies under (i), to identify genes regulated during prostate cancer progression; and to use cDNA to the C4-2B MF RNA to probe DNA microarrays, to identify genes differentially regulated in the C4-2B cells, that possess the targeted properties.

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INTRODUCTION

This project is directed to the problem of androgen-independent prostate cancer, which is a major cause of cancer-related mortality among men. The purpose of the research is to clone, isolate and characterize protein markers that are specifically expressed by advanced stage, androgen-independent prostate cancer.

BODY

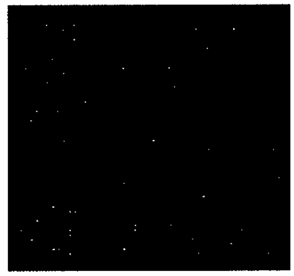
I. Incorporation of DNA Microarray Technology into the Project. In my Annual Progress Report of August, 1999 (p. 6), I described our plans to incorporate two new technologies into our research on the goals of this grant: DNA Microarray Technology and Proteomics. We have not made progress in our Proteomics approach, largely because of technical problems encountered by our collaborator in that work, Dr. Glucksman. However, we have now successfully introduced DNA Microarray Technology into this project. We are beginning to use this technology to identify genes differentially expressed among the four human prostate cancer cell lines described in the original application and the previous Annual Report (the cell lines are, in order of increasing independence of androgens and/or bone cells, and ability to exhibit bone metastasis: LNCaP, C4, C4-2, and C4-2b). Our results to date are summarized below.

We purchased from New England Nuclear their MICROMAX microarray system, which includes microarrays containing 2400 known human genes, plus reagents for probe preparation. Use of cDNA probes labeled with either Cy3 or

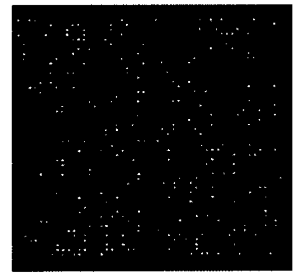
Cy5 permits simultaneous hybridization and analysis on one microarray of probes from two cell lines. The use of reference probes in each hybridization permits normalization of results between experiments. For each cell line, we have isolated total RNA, and performed at least two separate hybridizations of a probe prepared from its RNA to a MICROMAX microarray, under conditions where results from each cell line can be normalized and compared. The result from one such experiment that has been analyzed in detail is shown below (**Figure 1**). In this experiment, gene expression levels were compared between LNCaP cells (Fig. 1A, DNP cDNA label/ Cy3 detection) and C42 cells (Fig 1B, biotin cDNA label/ Cy5 detection). Fig. 1C shows a calculated superposition of the results with the two probes. Following normalization for the two-fold greater mean overall signal yielded by Cy5 relative to Cy3 in this (and most other) experiments, the scatterplot comparing individual gene expression levels shown in Fig.1D was obtained. It can be seen in Fig. 1D that a number of genes are differentially regulated between the two cell lines. Of the 2400 cDNAs present within the NEN microarray, 41 (1.71%) were found to be expressed at significantly higher levels in the LNCaP cell line, while 54 (2.3%) were found to be significantly up-regulated in the C42 cells. To my knowledge, these results represent the first report of the use of microarray technology to investigate differential gene expression in the LNCaP series of human prostate tumor cells, and demonstrate the great potential utility of this technology for detecting and

Figure 1. Microarray Analysis of Differential Gene Expression between LNCaP and C42 cell lines. 1A) TIF file of original Cy3 scan showing hybridization of DNP-labeled LNCaP cDNA. 1B) TIF file of original Cy5 scan showing hybridization of biotin-labeled C42 cDNA. 1C) ImaGene™ superimposed file of Cy3 and Cy5 scanned images.

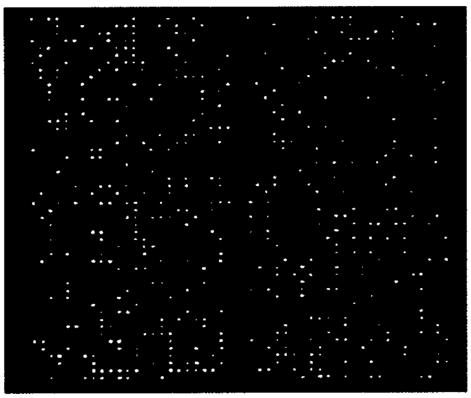
1A



1B



1C

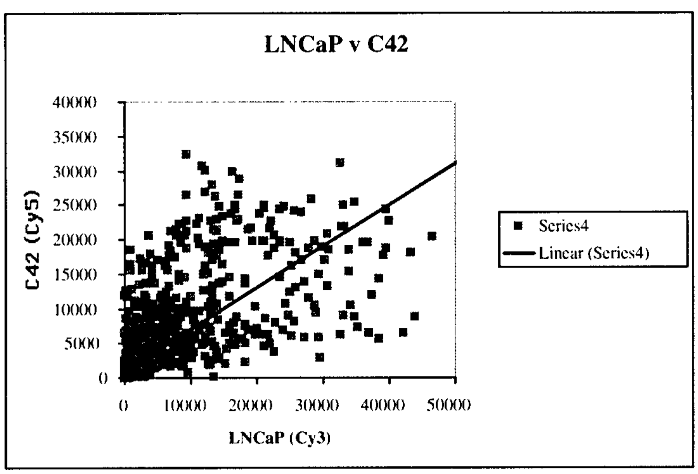


COLOUR CODE KEY:

Original TIF images.	Superimposed TIF images.
Blue- Green- Yellow- Red-White	Red Cy3 > Cy5
	Yellow Cy3= Cy5
Transcript Levels	Green Cy3< Cy5

Superposition of TIF images in 1A and 1B above, prior to normalization.

1D



Scatterplot of Cy3 (X axis) versus Cy5 (Y axis) signal intensities following normalization.

analyzing multiple changes in gene expression in the prostate cancer progression represented by this series of cell lines. We now plan to analyze our results with all of the cell lines in detail, in order to identify genes whose expression increases (or decreases) during the tumor progression represented by these cell lines.

II. Analysis of Membrane and Post-Membrane Fractions from the LNCaP Cell Series. As described in the previous Annual Report, we have prepared membrane and post-membrane fractions from the LNCaP cell line and two of its sub-lines (C42 and C4-2B). We then employed RT-PCR to prepare cDNA from RNA isolated from each fraction. To begin to test the utility of our fractions for further investigations, we employed semi-quantitative PCR of this cDNA to examine the presence in each fraction of each of four potential prostate cancer markers that have been described in the literature: the secreted marker PSA (Urol. Clin. North. Am., Vol 24); the cell-surface marker prostate-specific membrane antigen (PSM) (Israeli et al, 1997); TIMP-2, a metalloproteinase inhibitor found in body fluids, suggesting it is a secretory protein (Gomez et al, 1997); and PAGE-1, a protein recently cloned by another group from the LNCaP series (Chen et al, 1998), which is believed to be expressed as a cell surface antigen. Our results were as follows: PSA mRNA was detected in all three cell lines, almost exclusively in the membrane fraction. Only C4-2 cells yielded high levels of PSM and TIMP-2, again almost exclusively in the membrane fraction. Expression of PAGE-1 was significant only in the C4-2 cells, where its mRNA was mainly in the membrane fraction.

These results were encouraging, since the mRNA's for the four markers were in the expected cellular fraction, the membrane fraction. However, it was necessary also to examine the degree to which the membrane fraction, as we have isolated it, is contaminated with the post-membrane fraction. The latter fraction should contain mRNAs for proteins that are intracellular, and thus neither secretory nor cell surface proteins, and would thus ultimately yield false positives in our assay. To investigate this potential problem, we have carried out a number of control experiments of the type described above, in which PCR of the cDNAs prepared from mRNA in each fraction was employed to determine the intracellular location of the mRNAs for the following proteins expected to be intracellular: the PSM alternative splice variant PSM' (PSM-prime), which lacks the transmembrane attachment signal sequence (Sai et all, 1995); the transcription factor PREB (Fliss et al, 1999); and the transcription factor CTCF, which maps to a region commonly deleted in prostate (and breast) cancers (Filippova et al, 1998). Unfortunately, we have found that the membrane fractions isolated from the LNCaP cell series by the technique originally proposed and employed yields significant contamination by some or all of these mRNAs. We have thus proceeded to employ an alternative membrane fraction purification technique involving sucrose gradient density ultracentrifugation (Mechler, B.M., 1987) to again isolate membrane fractions from members of the LNCaP series, and then used PCR analysis of the mRNA for the transcription factor CTCF to analyze the purity of these fractions. This modified procedure has yielded variable success with the cell lines examined, as follows. The best results were

obtained with the cell line corresponding to the most advanced prostate cancer stage, the C4-2B cells. With these cells, PSA and TIMP-2 mRNA were again enriched in the membrane fraction; while CTCF mRNA was present in the post-membrane fraction, but was virtually absent from the membrane fraction. The purity of membrane preparations from the C4-2 cells was improved, but these preparations still contained slight contamination with CTCF mRNA. With the LNCaP cells, introduction of this new procedure was found to yield no improvement in the previously detected contamination of the membrane fraction with CTCF mRNA.

The membrane fraction from the C4-2B cells is expected to contain mRNAs that are both expressed in advanced stage prostate cancer, and encode proteins that are potentially clinically useful. We thus propose now to employ this fraction in DNA microarray experiments of the type described in Part I of this section. Briefly, we will prepare a probe from mRNA isolated from the membrane fraction of the C4-2b cells, hybridize it to the DNA microarrays described above, and compare the results to those we have already obtained with probes prepared from total cellular RNA from the cell lines (described in Part I above). Spots that hybridize with the C42b membrane fraction probe should represent genes encoding secretory or cell surface proteins, while the results of the investigations in Part I should tell us which of these genes exhibit changes in gene expression during the tumor progression represented by the LNCaP cell series. This approach should form the basis for a choice of genes to be further analyzed for their clinical utility.

KEY RESEARCH ACCOMPLISHMENTS

- Incorporated the powerful new DNA Microarray technology into project research, and used it to begin to identify genes differentially expressed among the LNCaP series of human prostate cancer cells. This is apparently the first study with DNA Microarray technology of differential gene expression among the LNCaP cell series.
- Discovered that membrane fractions previously isolated from these cells exhibited potential utility, but were of insufficient purity for proposed use. Employed an alternative cellular fractionation scheme, which yielded sufficiently pure membrane fractions from cells (C42-B) representing the most advanced stage of autonomy, but not from the other cell lines examined.

REPORTABLE OUTCOMES

When analysis of the results obtained with DNA Microarray technology described in Part I of the BODY is complete, a manuscript describing these results will be prepared.

CONCLUSIONS

During the reporting period, we have helped to establish at Mount Sinai the new but technically complex DNA Microarray technology, and have succeeded in employing this powerful technology to pursue the overall goals of

this project. Thus, as described above, we have employed this technology to carry out what is apparently the first study of differential gene expression among the LNCaP series of human prostate cancer cells. The expected outcome of this work, when our analysis of the results is complete, is the identification of a number of genes whose expression is differentially regulated during the stages of prostate cancer progression modeled by the LNCaP cell line and its derivatives.

We were disappointed when further control studies indicated that the mRNAs in the membrane fractions we had previously isolated from these cell lines, although enriched for mRNAs for known prostate cancer markers, were also contaminated with post-membrane fraction mRNAs. However, we have now employed an alternative fractionation scheme to isolate a highly pure membrane fraction from the C4-2b cells, which model advanced stage prostate cancer. As described above in the BODY of this report, this more highly purified membrane fraction should now be useful as a source for preparation of a DNA microarray hybridization probe specific for mRNAs encoding either secreted or cell-surface proteins. The results obtained with this probe, when compared to the results of the microarray experiments we have already carried out, should permit identification of genes that may prove clinically useful in diagnosis/treatment of advanced stage prostate cancer.

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